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Award Number: DAMD17-97-1-7274

TITLE: Muc1 Facilitation of Growth in Chemically Induced Mammary Gland Tumors in Muc1 Mutant and MUC1 Transgenic Mice

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REPORT DATE: August 1999

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 1999	Annual (1 Aug 98 - 31 Jul 99)

4. TITLE AND SUBTITLE
Muc1 Facilitation of Growth in Chemically Induced Mammary Gland Tumors in Muc1 Mutant and MUC1 Transgenic Mice

5. FUNDING NUMBERS
DAMD17-97-1-7274

6. AUTHOR(S)
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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, MD 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

MUC1 is a cell-associated mucin glycoprotein that is highly over-expressed in greater than 90% of mammary gland carcinomas. Our hypothesis is that the high level of expression of MUC1 in tumors facilitates tumor progression and metastasis. To generate spontaneously occurring tumors, we have mated mice carrying the unactivated c-neu protooncogene under control of the mouse mammary tumor virus (MMTV) LTR with Muc1 wildtype (+/+) or knockout (-/-) mice. These mice develop mainly unifocal tumors at about 7 to 12 months of age. Surprisingly, age at the onset of tumor growth was significantly lower for the Muc1-deficient mice than for the wild type mice ($p = 0.02$ from two-sided, two-sample t-test ($t=2.4$, $df = 34$). However, once tumors were palpable, the rate of tumor progression was similar in both groups. Muc1-deficient mice are twice as likely to have lung metastasis than Muc1 +/+ mice (39% vs. 18%). We found that the immune system of the Muc1 -/- mice was severely compromised, as T cells showed virtually no proliferative response to Concanavalin A or T-cell receptor stimulation. Although data are incomplete at this time, Muc1 has a dramatic effect on tumor latency, metastasis and general immune competence.

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES
12

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

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Sandra J. Gruder Aug. 25, 1999

PI - Signature Date

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Introduction

MUC1 is a cell-associated mucin glycoprotein that is highly over expressed in greater than 90% of mammary gland carcinomas. The high level of expression in carcinomas and metastatic lesions suggests an important role in tumor progression and metastasis. Our hypothesis is that MUC1 is a multi-functional protein and that several structural features contribute to its ability to modulate tumor progression and metastasis. These structural features include the large rodlike extracellular domain that extends far out from the cell surface and in all likelihood modulates the adhesiveness of cells (cell-cell and cell-matrix) and the vulnerability of the tumor cells to immune effector cells. The second structural feature of significance is the cytoplasmic tail domain that is phosphorylated and interacts with other phosphorylated proteins and with cytoplasmic proteins that may be involved in signal transduction. Our aims, as modified in the first annual report (August 1998), were to induce tumors in Muc1-deficient and wild type mice by mating them with mice carrying the unactivated c-neu protooncogene driven by the mouse mammary tumor virus long terminal repeat (MMTV- LTR). We further proposed to examine the effects of T cells and NK cells on tumor development and metastasis in these mice and to examine the characteristics of mammary tumor cells derived from the Muc1 -/- and +/+ mice to recognize and bind to various extracellular matrix ligands. This study is still in progress, but it has already yielded significant and surprising results.

Annual Summary

In order to utilize the neu tumor model to test the effect of Muc1-deficiency on tumor latency, progression, and metastasis, we had to backcross the Muc1-null animals onto the FVB strain, as the C57BL/6 strain has genes that modify neu tumor occurrence [1]. The backcrossing has taken nearly two years to accomplish. We have gone through 8 backcrosses of the Muc1 knockout (KO) mice onto inbred FVB mice, which should result in animals that are 99.61% FVB. Neu tumors are physiologically relevant, as about 30% of human breast cancers overexpress neu [2]. Neu tumors develop between 7 and 12 months of age and are unifocal rather than multifocal, in general; metastasis occurs in a large percentage of animals [3].

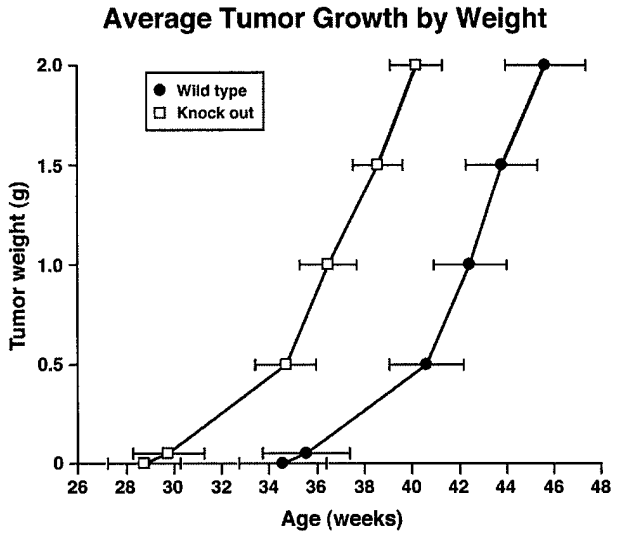

Fig 1. Age at onset of mammary tumors induced by the c-neu protooncogene is accelerated in mice lacking Muc1. Error bars represent standard error. Age at the onset of tumor growth was significantly lower for the Muc1 knockout mice (mean = 28.8 weeks, s = 6.0 weeks, n = 16) than for the wild type mice (mean = 34.6 weeks, s = 8.1 weeks, n = 20). The difference between the mean ages at onset of tumor growth was 5.8 weeks (95% CI = 0.9 to 10.7 weeks), and had P = 0.02 from the two-sided, two-sample t-test (t=2.4, df = 34)

We have nearly 120 animals enrolled in this study (in order to achieve statistical significance) and are presently palpating tumors in about 40 mice in each arm (Muc1 -/- and Muc1 +/+, each with the neu oncogene). Age at the onset of tumor growth was significantly lower for the Muc1 knockout mice (mean = 28.8 weeks, s = 6.0 weeks, n = 16) than for the wild type mice (mean = 34.6 weeks, s = 8.1 weeks, n = 20). The difference between the mean ages at onset of tumor growth was 5.8 weeks (95% CI = 0.9 to 10.7 weeks), and had P = 0.02 from the two-sided, two-sample t-test (t=2.4, df = 34) (Fig. 1). However, the rate of tumor progression, once the tumor reaches palpable size, appears to be similar in both groups. Muc1-deficient mice are twice as likely to have lung metastasis than Muc1 +/+ mice (39% vs. 18%). One possible interpretation of these results is that the immune system may have an effect on tumor progression at a time when the numbers of tumor cells were small, but once the tumors reached a sufficiently large size to be palpable, tumor proliferation overcame the capacity of the immune system to respond effectively.

To determine the general immunocompetent state of the immune system in these two groups of mice, a T cell blastogenesis assay was performed. Spleen, thymus, peripheral blood lymphocytes and tumor infiltrating lymphocytes were prepared and analyzed for ^{3}H-thymidine incorporation following stimulation with a limiting dilution of Concanavalin A ((ConA) 1 μg/ml down to 0.0625 μg/ml) (Fig. 2). Whereas the T cells from the Muc1-expressing mice were fully capable of proliferating following a six-day stimulation with ConA, the T cells from the Muc1-deficient mice showed virtually no proliferation. Similar results were obtained when T cells were stimulated more specifically with antibodies for CD3ε and CD28.

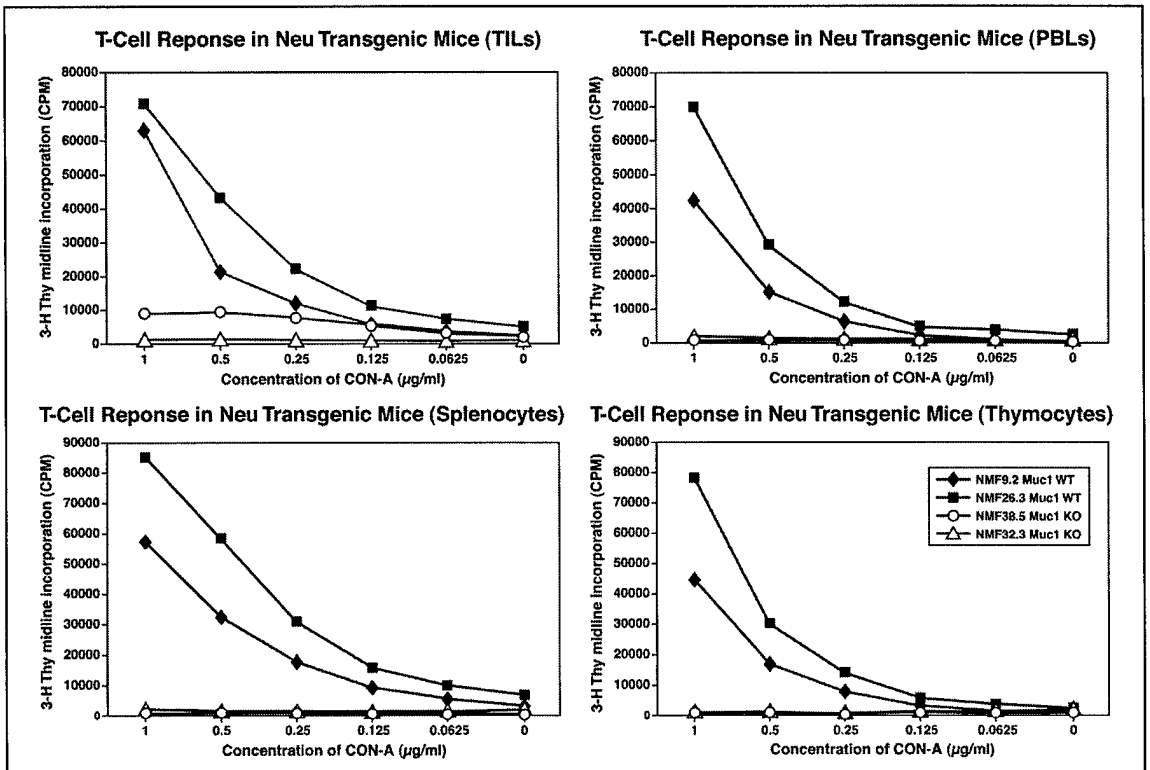

Fig. 2. T-cell blastogenic response in neu Muc1 +/+ or Muc1 -/- mice.

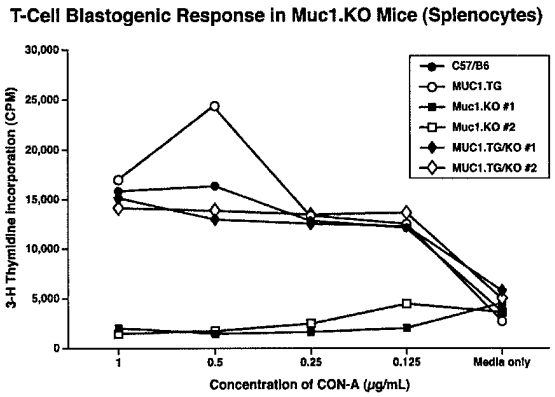

Fig. 3. T cell proliferation in Muc1 ko, wt, and MUC1.Tg on Muc1-/- background.

To determine that this effect is due to the lack of the Muc1 gene and not to a mutation that occurred elsewhere in the genome during the generation of the Muc1 -/- animals, we tested proliferation in human MUC1 transgenic mice on the Muc1 -/- background. The MUC1 transgenic line of mice was created using a 10 kb piece of human DNA containing the entire human MUC1 gene using its own endogenous promoter (called MUC1.Tg). We have shown that expression is tissue specific and copy number-dependent, i.e., the transgenic mouse does not overexpress human MUC1, but rather expresses it in a physiological manner [4]. When T-cell blastogenesis was assayed in MUC1.Tg mice on the Muc1-deficient background, the human MUC1 gene was found to compensate for the loss of mouse Muc1. Uptake of ^{3}H-thymidine was observed in wild type (Muc1-expressing) and MUC1.Tg mice lacking mouse Muc1 but not in Muc1-deficient mice (Fig. 3).

The failure of the T cells from the Muc1-deficient mice to proliferate was a very surprising result. To more fully assess this phenotype, T cell development was examined. T cell development is characterized by sequential steps that can be easily defined by the expression of particular cell surface proteins. Most T cells develop in the thymus through an ordered maturation process, giving rise to mature T cells with antigen-specific T cell receptors (TCRs). The earliest immature T cells lack expression of CD4 or CD8 and are called double negative (DN). DN thymocytes express the TCR signaling subunits TCRζ and the CD3 chains, but remain negative for mature TCR until rearrangement of the TCR (γ,δ,β) genes begins. Rearrangement of the β chain allows the TCRβ chain to associate with the invariant α chain to form a pre-TCR complex. Signaling through the pre-TCR allows the differentiation of DN cells to the CD4+CD8+ (double positive [DP]) stage, where rearrangement of TCRα then leads to expression of the mature form of the αβTCR. DP, αβTCR+ thymocytes undergo a number of TCR-mediated selection events and ultimately differentiate into either CD4+ or CD8+ mature single-positive (SP) T cells. Surprisingly, thymocyte development in the Muc1(-/-) mice showed an early arrest in the DN population. Total thymocytes from Muc1(-/-) or Muc1 (+/+) mice were analyzed for expression of CD4, CD8, CD3ε and a variety of other markers. Flow cytometric analysis revealed that DP thymocytes were largely absent in the Muc1-deficient mice (3%) compared to wildtype mice (76%) (Fig. 4a,b). Decreased levels of CD3ε, CD3ζ, CD28, CD25, TCRαβ and γδ, intracellular IL-2, IL-4 and IFN-γ were also observed.

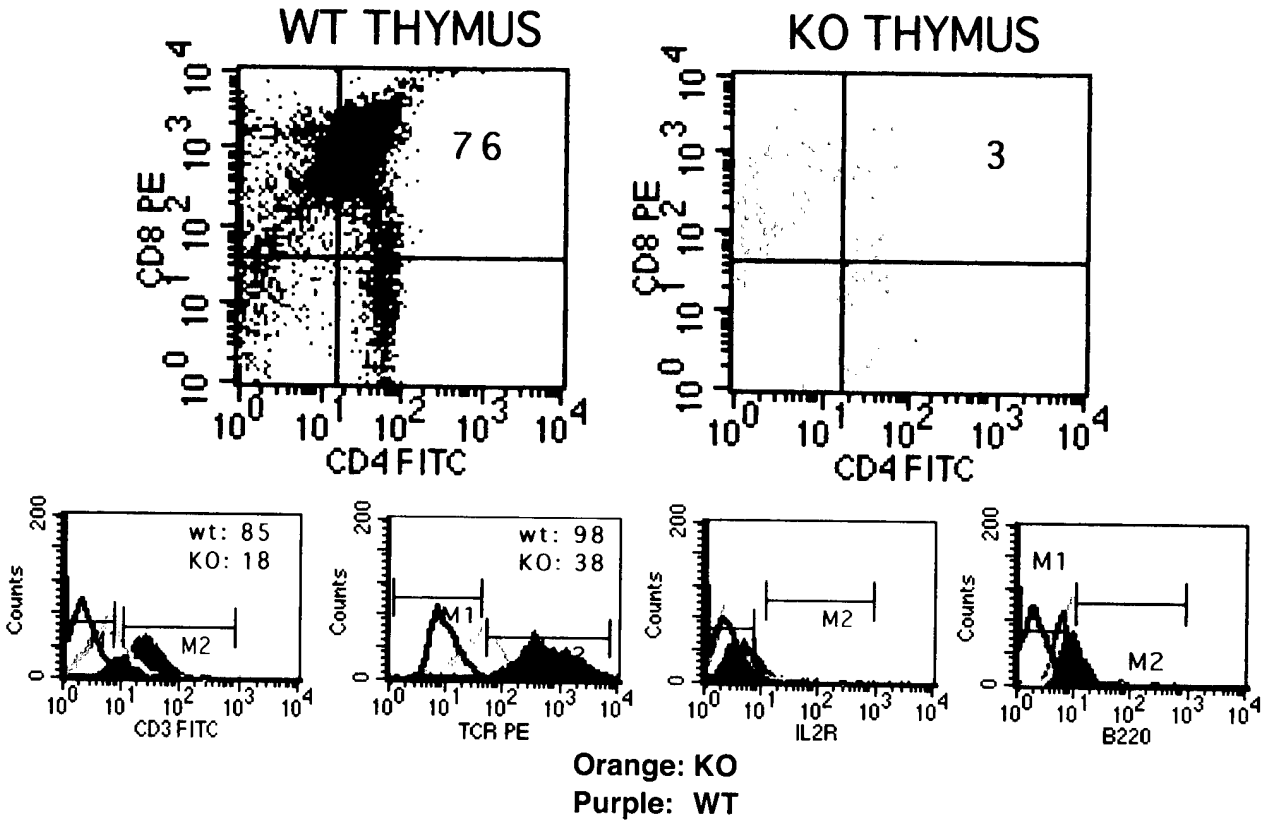

Fig. 4. Flow cytometric analyses of thymocytes from Muc1 -/- and Muc1 +/+ mice. Thymocytes from 6 to 8 week old adult mice were prepared, surface stained, and analyzed by standard flow cytometry. Two-color plots show staining of total thymocyte cells with antibodies against CD4 and CD8α (top row). The top two panels show DP thymocytes make up 76% of the cell population, whereas in the Muc1 -/- mice the DP population is only 3%. Single color-plot (bottom row) shows staining of thymocytes with α-CD3ε, α-TCRβ, α-IL2R, and α-B220 (purple = Muc1 +/+; yellow = Muc1 -/-; black = isotype control antibody). Numbers in quadrants indicate percentage of gated cells within the quadrant. Alterations in CD3 and TCR were observed.

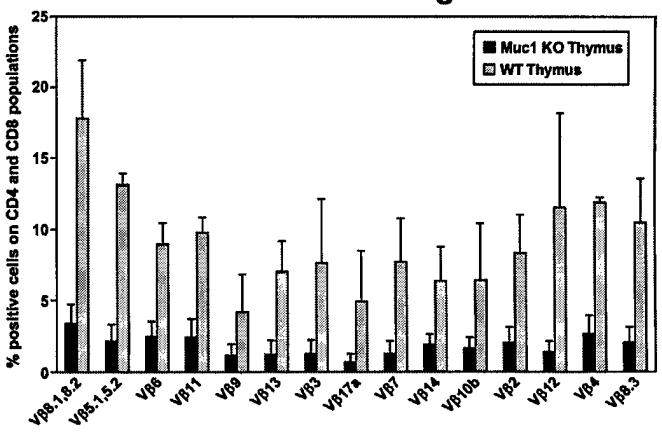

Fig. 5. TCR Vβ chain expression in WT and KO mice. Flow cytometric analysis of Vβ chain expression among CD4+ and CD8+ thymocytes. Error bars indicate the standard deviation.

Using a panel of antibodies reactive with a number of different TCR Vβ chains, we determined that WT mice had rearranged the beta chain on thymocytes, whereas the Muc1 KO mice did not appear to have rearranged the beta chain or else very low levels were expressed (Fig. 5). This result suggested that thymocyte development was arrested early in the DN stage of development.

Characterization of immune defects In this last year of this grant we will characterize these results more fully by determining if other lymphocytic subsets are affected by the Muc1 knockout, such as B cells, Natural Killer (NK) cells, and dendritic cells. We will use lipopolysaccharide (LPS) to determine if B cells are functional, perform NK cell assays using YAC cells as targets, and immunophenotype other cell types. Assays will be performed on any cell type shown to be compromised. We will test if the Muc1-deficient mice can reject allogeneic tumors or skin grafts. Studies characterizing B cells, NK cells, and dendritic cells will be performed on Muc1 +/+ and Muc1 -/- mice. Due to the length of time it takes to generate neu tumors, it is not feasible to perform such studies in tumor-bearing mice.

These studies are incomplete at this time, as tumor development in the neu mice occurs from about 7 to 12 months of age and proceeds for about ten additional weeks. The statistical analysis provided in this report is preliminary, as it was based on a partial cohort of mice enrolled in the study and was performed in order to complete this annual report. Once all the mice have reached 18 months of age, we will close the study and analyze a number of variables in addition to latency and metastasis. These include growth rate, time to onset (latency), length of tumor exposure (number of days between diagnosis of primary tumor and sacrifice at 2 gram size), metastasis, length of tumor exposure vs. density of pulmonary metastases, unifocality vs. multifocality of tumors, and expression of Muc1 and neu on tumors and metastatic lesions. The effect of a Muc1 deficiency on T cell development is a very recent and most unexpected finding. Muc1 has long been thought to be an epithelial-specific protein. It has been found only very recently that human T cells express human MUC1 [5]. We have recently determined that T cells in the mouse also express mouse Muc1 [6]. The observation that the immune system is defective in the Muc1 -/- mice originated from these studies. This work is providing us with additional knowledge of the function of the Muc1 mucin during tumorigenesis and progression. Muc1 is an important tumor antigen that appears to have profound effects on the immune system. Additional knowledge of this effect will enable us to better understand the biology of mammary gland adenocarcinomas.

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Appendix

1) Key Research Accomplishments

- Generation of mammary gland tumors in Muc1 +/+ and Muc1 -/- mice (120 mice enrolled in ongoing study)
- Statistically significant increase in age of onset of tumors in Muc1 -/- mice
- Growth rate similar in Muc1 +/+ and Muc1 -/- mice once tumors are of palpable size
- Increased incidence of lung metastasis in Muc1 -/- (39%) compared to Muc1 +/+ (19%) mice
- Severely compromised immune system in Muc1 -/- mice, as T cells fail to proliferate to either Concanavalin A or anti-CD3 and anti-CD28 stimulation
- Human MUC1 gene compensates for the deletion of mouse Muc1 gene in T cell proliferation assay in MUC1 transgenic mice
- T cell receptor beta chain failed to rearrange in Muc1 -/- mice
- Thymocyte development arrested in CD4/CD8 double negative stage

2) Reportable Outcomes

This work is not complete at this time, thus no reports have been made publicly. The postdoctoral fellow, Dr. Russell J. Vanderboom, left my laboratory to take a job as a Science Writer at The Cleveland Clinic. I am temporarily assuming this grant to ensure completion of this project.

3) Not Applicable